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Chaetocapnodium zapotae sp. nov. on *Manilkara zapota* in central Mexico

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Abstract–A new sooty mould, *Chaetocapnodium zapotae,* was isolated from *Manilkara zapota* in central Veracruz, Mexico. An analysis of ITS+LSU nuclear rDNA concatenated sequences of our isolate revealed taxonomic identity at the genus level located in the same clade as *Chaetocapnodium insulare* and *Chaetocapnodium placitae*. Morphological examination confirmed that the new species differs from *C. insulare* in the absence of the sexual morph and the absence of setae on the pycnidia. Additional characters distinguishing *C. zapotae* from *C. placitae* are its narrower pycnidial size range, wider size ranges for the hyphae and conidia, and the dark brown color of its pycnidia.

Key words–*Capnodiaceae*, sapodilla fruit, *Sapotaceae*, sooty mould

Introduction

Manilkara zapota (sapodilla) is a tropical crop native to southern Mexico and Central America that is particularly valued for its anti-inflammatory, antioxidant, antimicrobial, antifungal, and anticancer properties (Khalek & al. 2015). Sapodilla orchards in central Mexico recently developed clear signs of disease involving black spots on the surface of the fruit, the cause of which

was identified as an unknown sooty mould (*Capnodiales* s.lat.). Approximately 200 species of sooty moulds have been described from many different hosts in tropical and subtropical forests. This group is characterized by the formation of a network of dark-coloured hyphae on the surface of fruits and leaves (Hughes 1976, Chomnunti & al. 2014, Hongsanan & al. 2015).

Recent extensive documentation of sooty moulds, including morphological descriptions, ecological studies, mode of nutrition, and nDNA ITS, LSU, RPB2 and TEF-1α sequence analyses have revealed the existence of a polyphyletic group covering great species diversity supported within two different classes (*Eurotiomycetes, Dothideomycetes*) and seven different orders (*Capnodiales*, *Mycosphaerellales*, *Comminutisporales*, *Cladosporiales*, *Neophaeothecales*, *Phaeothecales*, *Racodiales;* Crous & al. 2007, Abdollahzadeh & al. 2020). *Capnodiales,* considered the second largest order within *Dothideomycetes* (Abdollahzadeh & al. 2020), which includes epiphyte fungi that grow on honeydew secreted by insects or plant exudates (Hughes 1976, Crous & al. 2009, Chomnunti & al. 2014), is represented by four families (*Euantennariaceae*, *Metacapnodiaceae*, *Antennulariellaceae*, *Capnodiaceae*). The placement of *Chaetocapnodium* in *Capnodiaceae* is based on the morphology of its sexual morph and supported by LSU sequence data (Liu & al. 2015). More robust phylogenies have since revealed that some species lacking the sexual morph are congeneric with *Chaetocapnodium*, such as *Antennariella placitae,* now recombined as *Chaetocapnodium placitae* (Abdollahzadeh & al. 2020).

The main objective of this study is the morphological and genetic identification of the fungus that generates sooty mould on the epidermis of sapodilla fruits in central Mexico.

Material & methods

Sapodilla fruits were collected during May–June 2017 and 2018 from 10–12 m tall *Manilkara zapota* trees in two ~1 ha orchards close to Apazapan village located in an area of sapodilla production in Veracruz state, Mexico. Fruit samples were labeled and taken to the laboratory in the Centro de Investigación en Micología Aplicada (CIMA), Universidad Veracruzana. The samples were washed with water, and black spots on fruits were cut into small pieces (0.5 cm^2), disinfected with 2% sodium hypochlorite for 30 seconds, and washed twice with sterile water. Groups of five fruit pieces, each group separated by 2 cm, were placed 2 cm apart in Petri dishes on potato Dibico dextrose agar (PDA) containing 0.2 g/L chloramphenicol. The Petri dishes were incubated at 25 ± 2 °C for 2–3 days until samples had developed mycelia. After growth, the mycelium was carefully transferred to a new PDA + chloramphenicol agar plate and incubated under similar conditions for fungal isolation.

Table 1. Species and sequences used for the construction of the phylogenetic tree. Type specimens are annotated as [T].

Species	Voucher	GenBank No.		Country
		ITS	LSU	
Capnodium alfenasii	CBS 146151 [T]	MN749233	MN749165	Brazil
C. blackwelliae	CBS 133588 [T]	MN749235	MH878118	USA
C. coffeicola	MFLUCC15-0206 [T]	KU358921	NG_068231	Thailand
C. gamsii	CBS 892.73 [T]	MN749237	GU301847	Sri Lanka
C. neocoffeicola	CBS 139614 [T]	MN749242	MN749172	Thailand
C. paracoffeicola	CBS 139616 [T]	MN749244	MN749174	Thailand
C. salicinum	CBS 131.34	MH855469	MH866941	Indonesia
Chaetocapnodium indonesiacum	CBS 202.30 [T]	MH855113	MH866561	Indonesia
Ch. insulare	CBS 146159 [T]	NR_168830	NG_068681	South Africa
Ch. philippinense	MFLUCC12-0110 [T]	NR_168831	KP744503	Philippines
Ch. placitae	CBS 124758 [T]	MH863403	MH874920	Australia
Ch. siamense	CBS 139815	MN749252	MN749181	Thailand
Ch. summerellii	CBS 146157 [T]	NR_168829	MN749176	Australia
Ch. tanzanicum	CBS 145.79 [T]	NR_168832	MN749182	Tanzania
Ch. thailandense	CBS 139619 [T]	NR_168833	MN749183	Thailand
Ch. zapotae	CM-CNRG 938 [T]	MW258620	MW258621	Mexico
Conidiocarpus asiaticus	MFLUCC10-0062	JN832597	JN832612	Thailand
Co. siamensis	MFLUCC10-0064 [T]	KU358926	JN832609	Thailand
Heteroconium citharexyli	HM628775 [T]	HM628776	HM628775	Ecuador
Leptoxyphium citri	CBS 451.66 [T]	MN749266	KF902094	Spain
L. glochidion	IFRDCC 2651 [T]	NR_155316	KF982308	China
L. kurandae	CBS 129530 [T]	JF951150	JF951170	Australia
L. madagascariense	CBS 124766 [T]	MH863407	MH874923	Madagascar
Microxiphium leptospermi	CBS 278.34	MH855514	MH867017	Australia
Phragmocapnias betle	MFLUCC10-0053 [T]	KU358922	JN832606	Thailand
Ph. plumeriae	MFLUCC15-0205 [T]	KU358919	NG_058933	Thailand
Polychaeton citri	CBS 116435	GU214649	GU214469	Iran
P. tenellum	CBS 201.30	MH855112	MH866560	Indonesia

The hyphae and reproductive structures produced on PDA were morphologically identified using a Zeiss 1122-100 Axiostar microscope at 1000× magnification. Samples were mounted on a microscope slide with a drop of lactophenol blue solution; hyphal width, pycnidial width and height, and conidial width and height

measured (n = 50 per character). Means and standard errors (SE) are provided for sizes; extreme values are given in parentheses. Samples were compared based on previously described characters (Cheewangkoon & al. 2009, Liu & al. 2015, Abdollahzadeh & al. 2020). The holotype specimen (CM-CNRG 938, dried agar plate culture) was deposited in the Microorganism Collection of the National Center for Genetic Resources (CM-CNRG) of the National Institute of Forestry, Agricultural and Livestock Research (INIFAP) in Mexico.

Genomic DNA was extracted from fresh mycelium according to Liu & al. (2000). Amplifications of the ribosomal internal transcribed spacer region (ITS) used ITS1F/ITS4 primers (White & al. 1990, Gardes & Bruns 1993) and of the ribosomal DNA large subunit (LSU) used LR0R/LR7 primers (Vilgalys & Hester 1990). DNA was amplified in a SureCycler 8800 thermal cycler under the following conditions: initial denaturation at 94 °C for 3 min, followed by 34 cycles at 94 °C for 45 sec, 53 °C for 45 sec (ITS primers) or 55 °C for 1 min (LSU primers), followed by 72 °C for one min and final extension cycle at 72 °C for 10 min. The amplicons were processed on a 1% agarose gel at 95 V for 60 min, and the gel was stained with ethidium bromide and photographed using the Gel Doc XR + Gel Documentation System. The amplicons were purified using the Wizard® SV Gel and PCR System Clean Up kit and sent to Labsergen Langebio for sequencing on an AB3770 capillary sequencer.

The sequences were compared in the GenBank nucleotide sequence database (Benson & al. 2017) using BLAST (Zhang & al. 2000) search software to confirm the genus and percentage identity. Closely related species sequences and our newly obtained sequences were incorporated into sequence datasets independently for each molecular marker using the PhyDE v.0.9971 Phylogenetic Development Editor (Müller & al. 2010). Each dataset was independently aligned using the MAFFT online service (Katoh & al. 2019). Inconsistencies were manually adjusted and the data set of concatenated ITS+LSU sequences was integrated. The IQ-Tree program (Nguyen & al. 2015) in an online interface (Trifinopoulos & al. 2016) was used to calculate the evolutionary model (Kalyaanamoorthy & al. 2017), using the Bayesian Information Criterion (BIC) to select the best fit model. A phylogenetic tree was generated using the Maximum Likelihood (ML) method with a Nearest Neighbor Interchange (NNI) heuristic algorithm and a TNe + G4 evolutionary model. Bootstrap analysis was performed with 1000 repetitions. The resulting phylogenetic tree was visualized using FigTree v1.4.3 (Rambaut 2016). The ITS and LSU sequences generated in this study were deposited in the GenBank database, under the accession numbers provided in the taxonomic section.

Taxonomy

Chaetocapnodium zapotae L. Navarro, Salinas & Trigos, **sp. nov.** Fig. 1

MB 837989

Differs from *Chaetocapnodium insulare* by the absence of a sexual morph and the absence of setae on the pycnidia.

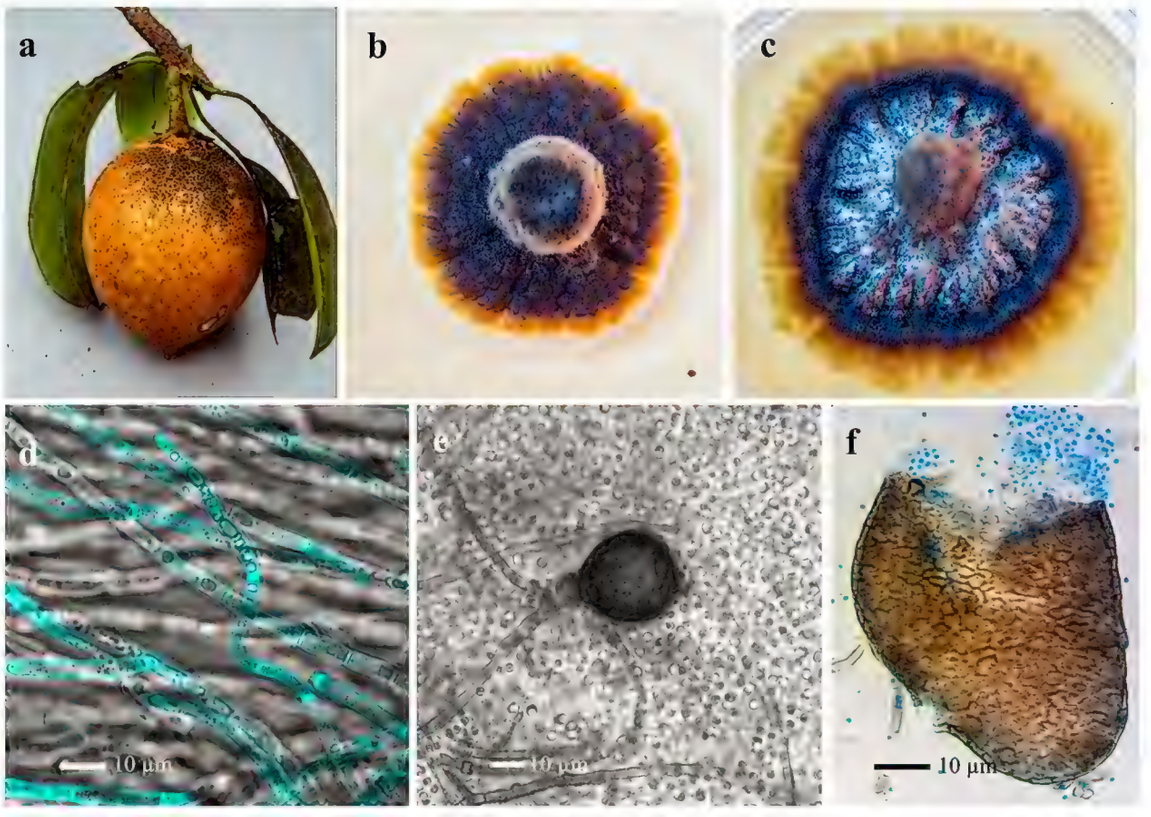

Fig. 1. *Chaetocapnodium zapotae* (holotype, CM-CNRG 938): a. Fruit of *Manilkara zapota* (sapodilla) with sooty mould on its surface; b. Mycelia grown in PDA medium for 2 wk at 25 °C in the dark; c. Mycelia cultured for 4 wks in the dark; d. Septate hyphae; e. Conidia and pycnidia; f. Irregular rupture of pycnidia releasing spores.

Type: Mexico. Veracruz State: Apazapan, 19.3381°N 96.7317°W, alt. 300 m a.s.l., on *Manilkara zapota* (L.) P. Royen (*Sapotaceae*), sapodilla fruit, Apr. 2018 (**Holotype**, CM-CNRG 938; GenBank MW258620, MW258621).

Etymology: zapotae, referring to the host plant species, *Manilkara zapota*, from which it was isolated.

Blackish fungus that grows on the exocarp surface of sapodilla fruit; Mycelium superficial or immersed, brown, septate, branched; Hyphae smooth, septate, 3.5 ± 0.1 µm wide (2–5 µm), initially hyaline, later dark brown, with a mucilaginous outer wall layer.

Pycnidia superficial or immersed, globose to subovoid, dark brown, 39.0 ± 1.7 × 32 ± 1.4 µm (21–72 × 18–58 µm), lateral, terminal or intercalary, thin-walled layers of textura angularis and a short neck; Ostiole absent or not well developed, mostly releasing conidia by means of irregular rupture at maturity; Setae not observed. Numerous conidia hyaline, globose to subglobose, with an average size 3.0 ± 0.1 × 2.5 ± 0.1 µm (2–4 × 2–4 µm), smooth and aseptate.

Sexual morph unknown.

Colonies leathery, dark brown, with fluffy aerial mycelium at the centre and creamy conidial exudates; edge sinuate with medium brown aerial mycelium after 3 wks in the dark at 25 °C; colonies reaching 12.6 mm diam on PDA after 2 wks in the dark at 25 °C.

Phylogenetic results

A phylogenetic tree was constructed with concatenated ITS+LSU sequences of 28 different species, with a final length of 1227 bp including gaps. Sequences of *Capnodium salicinum* CBS 131.34, *Microxiphium leptospermi* CBS 278.34, and *Polychaeton tenellum* CBS 201.30 were selected as outgroup (Table 1, Fig.2). Phylogenetic tree branches are indicated with their respective bootstrap values (BS) and the Bayesian posterior probabilities (BPP). Our phylogenetic tree strongly supports (100/1) our isolate as an independent species in *Chaetocapnodium* and sister to *C. insulare* and *C. placitae* (Fig. 2).

Discussion

Two other *Chaetocapnodium* species, *C. insulare* Abdollahz. & Crous and *C. placitae* (Cheew. & Crous) Abdollahz. & Crous are genetically related to *C. zapotae*. Although they share common morphological characteristics within the genus, we observed unique characteristics that support *C. zapotae* as a separate species. *Chaetocapnodium insulare* is distinguished by superficial or immersed pycnidia that are globose to pyriform, pale to dark brown, and generally small (28–)35–55 × (22–)30–48 µm; its pycnidia present setae [septate or aseptate, pale to dark brown, (7–)10–13(–19) µm] mostly around the ostiole; and its conidia are hyaline, aseptate, globose to subglobose, and longer and wider [av. = 3.4 × 3 µm; (2.8–)3.2–3.6(–4.4) × (2.6–)2.9–3.3(–3.7) µm; (Abdollahzadeh & al. 2020)] compared to other *Chaetocapnodium* species; it also presents ascomata with 3-septate brown ascospores not observed in *C. zapotae*. The radial growth rate on MEA at 25 °C is considered slow (14 mm diam/2 wk) compared to other *Chaetocapnodium* species (Abdollahzadeh & al. 2020). Nonetheless its radial growth is higher than observed for *C. zapotae* (12.6 mm diam on PDA after 2 wk in the dark at 25 °C).

The main differences between *C. zapotae* and *C. placitae* are the pycnidial coloration (brown (Fig. 1b), not medium to dark grey-brown) and a narrower pycnidial size range (21–72 × 18–58 µm; Fig. 1e, f) vs. (30–)40–60(–120) × (22–)30–40(–65) µm previously described for *C. placitae* (Cheewangkoon & al. 2009). In culture, *C. placitae* colonies exhibit an entire edge with medium to dark brownish grey woolly aerial mycelium contrasting with the sinuate

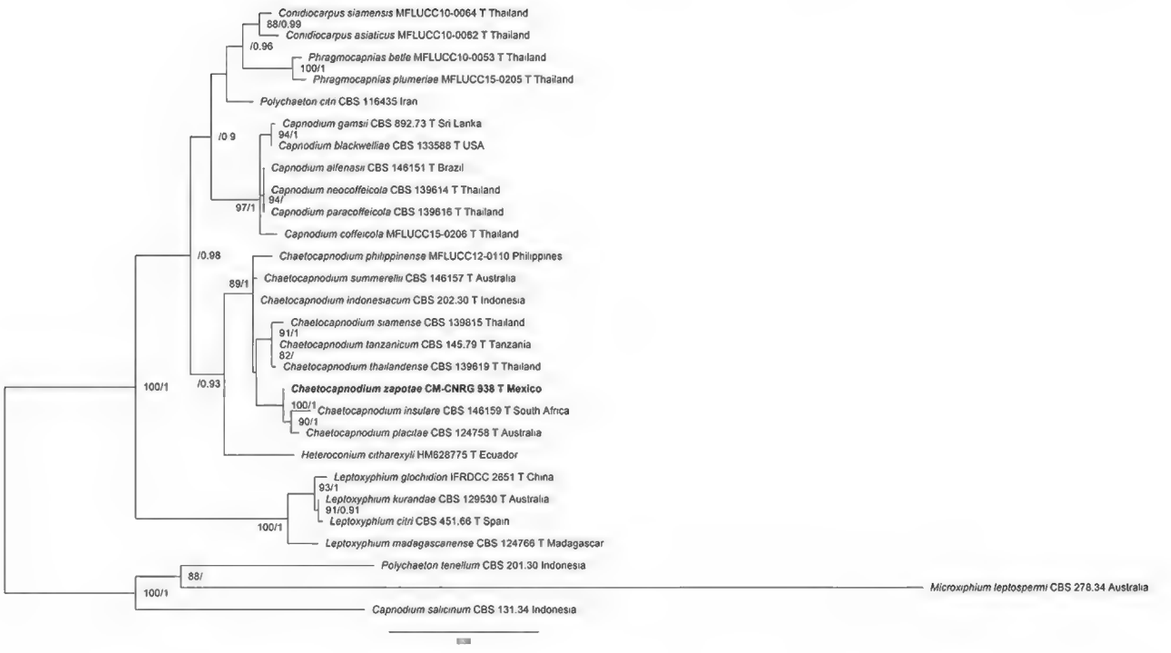

Fig. 2. Maximum likelihood phylogenetic tree generated from the analysis of the concatenated ITS+LSU sequences of the *Chaetocapnodium zapotae* (holotype, CM-CNRG 938), and closely related species downloaded from GenBank. The numbers at the nodes indicate Bootstrap values for 1000 replicates (BS ≥70%)/Bayesian Posterior Probabilities (BPP ≥0.9).

edge with medium brown aerial mycelium observed in *C. zapotae* (Fig. 1c). *Chaetocapnodium placitae* shows a more restricted size range for hyphae (range 3.5–5 µm) and conidia (2.3–)2.5–3(–3.8) × (2–)2.5–2.8(–3.2) µm when compared with *C. zapotae* hyphae (Fig. 1d). The radial growth rate of *C. placitae* on MEA at 25 °C is 20 mm diam/2 wk (Cheewangkoon & al. 2009), considerably higher than observed for *C. zapotae* in PDA. Although sexual morphology is an important characteristic within *Chaetocapnodium* (Liu & al. 2015), the sexual morph is unknown in both *C. placitae* and *C. zapotae*. However, the robust phylogeny by Abdollahzadeh & al. (2020) supports *C. placitae* in *Chaetocapnodium* despite its unknown sexual morph. and includes *C. placitae* and *C. indonesiacum* as the only two *Chaetocapnodium* species lacking setae on the pycnidia (Abdollahzadeh & al. 2020). Our phylogeny adds *C. zapotae* as a third species lacking pycnidial setae.

Our phylogenetic and morphological analyses confirm that the species is not *C. insulare* due to the absence of the sexual morph and the absence of setae on the pycnidia. Although *C. placitae* shares some similarities with our isolate, such as the absence of the sexual morph and the absence of setae on the pycnidia, *C. zapotae* is distinguished by its smaller dark brown pycnidia and its greater hyphal width and conidial width and length ranges. These

morphological differences and our new phylogeny support this isolate as a new species, which we name *Chaetocapnodium zapotae*.

We conclude that *C. zapotae* represents a new species that produces black spots on sapodilla fruits (Fig. 1a) and leaves in central Veracruz State, Mexico. This is the first report of a *Chaetocapnodium* species associated with sapodilla.

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